Surface Protein Composition of *Aeromonas hydrophila* Strains Virulent for Fish: Identification of a Surface Array Protein

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The surface protein composition of members of a serogroup of *Aeromonas hydrophila* which exhibit high virulence for fish was examined. Treatment of whole cells of representative strain *A. hydrophila* TF7 with 0.2 M glycine buffer (pH 4.0) resulted in the release of sheets of a tetragonal surface protein array. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis showed that this sheet material was composed primarily of a protein of apparent molecular weight 52,000 (52K protein). A 52K protein was also the predominant protein in glycine extracts of other members of the high-virulence serogroup. Immunoblotting with antiserum raised against formalinized whole cells of *A. hydrophila* TF7 showed the 52K S-layer protein to be the major surface protein antigen, and impermeant Sulfo-NHS-Biotin cell surface labeling showed that the 52K S-layer protein was the only protein accessible to the Sulfo-NHS-Biotin label and effectively masked underlying outer membrane (OM) proteins. In its native surface conformation the 52K S-layer protein was only weakly reactive with a lactoperoxidase ^{125}I surface iodination procedure. A UV-induced rough lipopolysaccharide (LPS) mutant of TF7 was found to produce an intact S layer, but a deep rough LPS mutant was unable to maintain an array on the cell surface and excreted the S-layer protein into the growth medium, indicating that a minimum LPS oligosaccharide size was required for *A. hydrophila* S-layer anchoring. The 52K S-layer protein exhibited heat-dependent SDS-solubilization behavior when associated with OM, but was fully solubilized at all temperatures after removal from the OM, indicating a strong interaction of the S layer with the underlying OM. The native S layer was permeable to ^{125}I in the lactoperoxidase radiolabeling procedure, and two major OM proteins of molecular weights 30,000 and 48,000 were iodinated. The 48K species was a peptidoglycan-associated, transmembrane protein which exhibited heat-modifiable SDS solubilization behaviour characteristic of a porin protein. A 50K major peptidoglycan-associated OM protein which was not radiolabeled exhibited similar SDS heat modification characteristics and possibly represents a second porin protein.

Aeromonas hydrophila, a gram-negative motile rod, is a pathogen of a wide variety of animals, including humans. The species is especially important as a pathogen of fish, typically producing a fulminant hemorrhagic septicemia. In many instances disease appears to result from opportunistic infection by strains of *A. hydrophila* in the normal flora of the fish. In other cases however, the strains of *A. hydrophila* producing fish disease clearly behave as primary pathogens. *A. hydrophila* is not normally regarded as a major pathogen of salmonid fish. However, one group of strains with high virulence for salmonids has been reported by Mittal et al. (28). These strains exhibit certain cell-surface-associated phenotypic characteristics unique among *A. hydrophila* isolates. They autoaggregate in static broth culture, they are resistant to the bactericidal activity of normal serum, and they form a single thermostable serogroup which we have previously shown to be based on the O-polysaccharide antigens of their lipopolysaccharides (LPS) (9). These O polysaccharides are of homogeneous chain length, in contrast to the heterogeneous chain lengths of O polysaccharides of other *A. hydrophila* strains.

A first step in pathogenesis for a systemic pathogen such as *A. hydrophila* must be colonization of the host. Many of the properties which facilitate the colonization event are associated with the bacterial cell surface. These bacterial properties can include the production of proteinaceous adhesins (21), certain outer membrane (OM) proteins (5), and

antiphagocytic carbohydrate capsules (32). A number of bacteria also produce paracrystalline arrays of proteins known as S layers on their surface. S layers are regular, two-dimensional assemblies of protein monomers that often constitute the outermost layer of the cell envelope of many bacteria (34). A variety of subunit arrangements in the form of hexagonal, tetragonal, and linear oblique arrays have been described (34). The information necessary for layer assembly is present in the primary structure of the protein subunits for entropy-driven assembly, although a suitable cell envelope template and divalent cations are usually required. The layers themselves are held together and attached to the underlying cell envelope by a variety of noncovalent interactions. The surface location of this protein structure means that for pathogens in vivo, the S layer must come into close contact with the cells and tissue fluids of its host. S layers are therefore ideally sited to influence the outcome of a host-parasite relationship, and, not surprisingly, S layers on pathogenic bacteria are being increasingly reported (27, 30). Indeed, for *Aeromonas salmonicida*, an important fish pathogen, the presence of a tetragonal S layer was shown to be essential for virulence (20).

In this paper we report that all members of the serogroup of *A. hydrophila* with high virulence for fish produce a tetragonally arrayed S layer. Using low-pH extraction, together with surface labeling and immunoblotting with polyclonal antiserum to intact *A. hydrophila* cells, we have identified the S layer protein of *A. hydrophila*. We also

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describe the composition and arrangement of the major proteins in the OM of S-layer-producing *A. hydrophila*.

MATERIALS AND METHODS

Bacterial strains. The *Aeromonas* strains studied and their sources are listed in Table 1. Stock cultures were maintained at −70°C in 15% (vol/vol) glycerol–tryptic soy broth (GIBCO Diagnostics, Madison, Wis.). *A. hydrophila* cultures were grown on tryptic soy agar (GIBCO) at 37°C for 18 to 24 h and harvested with a glass microscope slide. *A. salmonicida* was grown in the same medium at 20°C for 48 h.

Electron microscopy. The best visualization of the S layer on whole cells or on isolated sheets was obtained by negative staining with either saturated ammonium molybdate (ca. 4%, pH 5.5) or 2% phosphotungstate (pH 6.8). Under these conditions, areas of S-layer material that sloughed off the cells and attached to the carbon–Formvar-coated grids were easily visualized. Magnification was calibrated with crystals of catalase negatively stained with 2% phosphotungstate. Micrographs were recorded in an EM300 electron microscope (Philips Electronics, Toronto, Ontario, Canada) operating at 80 kV.

Isolation of S-layer sheets. Cells were harvested after 24 h of growth and washed three times in 20 mM Tris (pH 8.0). They were suspended in 0.2 M glycine (pH 4.0) at 3 g of cells per 100 ml of buffer and stirred at 4°C for 15 min. The cells were removed by three sequential centrifugations at 12,000 × g for 20 min. The S-layer sheet material was collected by centrifugation at 40,000 × g for 30 min and washed once in Tris buffer.

Isolation of OMs. *A. hydrophila* cells were harvested and washed once in 20 mM Tris hydrochloride (pH 7.4). Cells (0.5 g [wet weight]) were suspended in 10 ml of ice-cold Tris buffer. DNase and RNase were added, and the cells were disrupted by three passages through a French pressure cell at 16,000 lb/in^2. Unbroken cells were removed by centrifugation at 6,000 × g for 30 min at 4°C. The total cell envelope fraction was isolated by centrifugation at 32,000 × g for 30 min at 4°C. OMs were prepared from isolated cell envelopes by differential solubilization of the inner membrane by the sodium lauryl sarcosinate method described by Filip et al. (13). Alternatively, the envelope pellet was suspended in 1.0 ml of 20 mM Tris (pH 7.4) made up of 10% (wt/wt) sucrose and layered on top of a discontinuous sucrose gradient made up of 2.5 ml each of 20%, 30%, 40%, 50%, and 60% (wt/wt)

TABLE 1. *Aeromonas* strains used

Strain	Source[a]
A. hydrophila	
TF7[b]	Trout lesion, Quebec (R. Lallier)
LL1[b]	Trout lesion, Quebec (R. Lallier)
A80-140[b]	Aborted piglet liver, Quebec (R. Lallier)
A80-160[b]	Bovine brain, Quebec (R. Lallier)
A82-256[b]	Moribund trout, Quebec (R. Lallier)
P77-115[b]	Otary lung, Quebec (R. Lallier)
TF7/U14	LPS mutant of TF7 (this laboratory)
TF7/B	LPS mutant of TF7 (this laboratory)
A. salmonicida	
449	Trout, France (C. Michel)

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[b] Strains shown to exhibit enhanced virulence for fish (28).

sucrose in 20 mM Tris (pH 7.4) that had been allowed to equilibrate for 4 h at 4°C. Gradients were spun for 22 h at 275,000 × g at 4°C. Fractions (0.5 ml) were collected from the bottom of the tube. The protein content was estimated by monitoring A_{280}, and NADH oxidase activity was determined as described by Osborn et al. (31). OM and S-layer protein were prepared from *A. salmonicida* as previously described (22).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (24). A model 360 Mini Vertical Slab Cell system (Bio-Rad Laboratories, Richmond, Calif.) was used for all separations. Samples were boiled for 10 min in SDS-PAGE solubilization buffer (10% glycerol, 5% β-mercaptoethanol, 3% SDS, 0.01% bromophenol blue in 62.5 mM Tris [pH 6.8]). Gels were run for 10 min at 100 V through 4.5% acrylamide stacking gels and for a further 35 min at 200 V through either 7.5% or 12.5% acrylamide separating gels. To visualize proteins, the gels were stained with Coomassie blue or the silver stain described by Wray et al. (37).

Analysis of LPS. The electrophoretic migration pattern of LPS was determined by a modification of the procedure of Hitchcock and Brown (17). Cells were boiled in the SDS-PAGE solubilization buffer for 5 min and then digested with proteinase K at 60°C for 1 h at a ratio of 10 mg of bacterial cell mass to 1 mg of proteinase K. Samples were loaded onto SDS-PAGE gels (12.5% acrylamide) at a concentration of 50 μg of original cell mass per lane. After electrophoresis, gels were silver stained by the procedure of Tsai and Frasch (36).

Polyclonal antiserum. Antiserum against formalinized cells of *A. hydrophila* TF7 was prepared in rabbits as previously described (10). Antibodies to heat-stable antigens were removed by absorption with a boiled suspension of the homologous strain as previously described (10).

Immunoblotting. Following SDS-PAGE, membrane components were transferred from the slab gel to nitrocellulose paper by using the methanol-Tris-glycine system of Towbin et al. (35). Electroblotting was carried out in a transblot apparatus (Bio-Rad) for 6 to 12 h at 60 V. Unreacted sites on the paper were blocked with a 1% (wt/vol) solution of gelatin in 10 mM Tris (pH 7.4)–0.9% NaCl (TSG buffer) for 1 h at room temperature. The nitrocellulose paper was then incubated with an appropriate dilution of antiserum in TSG buffer for 2 h. After being washed in 10 mM Tris buffer (pH 7.4)–0.9 (wt/vol) NaCl–0.05% (vol/vol) Tween 20 for 1 h with three changes of solution, the sheets were incubated with ^{125}I-labeled protein A (50,000 cpm/ml) for 2 h in TSG buffer. This was followed by a 1-h wash in Tris buffer, and bound radiolabeled protein A was detected by autoradiography of the dried sheets for 24 h with X-Omat AR-5 film (Eastman Kodak Co., Rochester, N.Y.).

Biotinylation of surface proteins. The surface protein biotinylation system was modified from the procedure of Hurley et al. (19). Cells were suspended in phosphate-buffered saline (PBS, pH 7.4) to a final concentration of 1 mg/ml (wet weight). Sulfo-NHS-Biotin (Pierce Chemical Co., Rockford, Ill.) in dimethyl sulfoxide (2 μl of a 50-mg/ml solution) was added, and the sample was incubated at room temperature for 2 min. The cells were washed three times with PBS and prepared for SDS-PAGE by being boiled in 100 μl of solubilization buffer for 10 min. After electrophoresis, gels were blotted onto nitrocellulose paper (pore size, 0.45 μm) by the Tris-glycine-methanol buffer system of Towbin et al. (35). After transfer, the nitrocellulose paper sheets were soaked in PBS–1% gelatin for 1 h at room temperature with constant agitation. Streptavidin-biotinylated horseradish
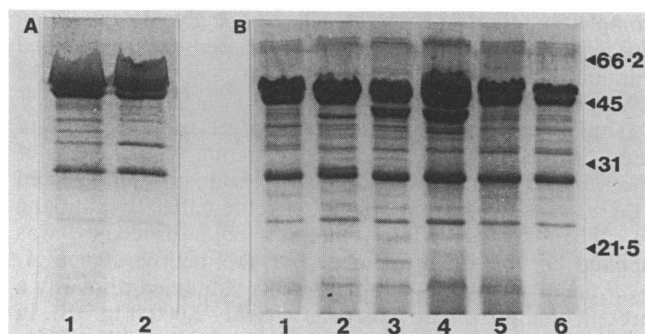
FIG. 1. SDS-PAGE (12.5% acrylamide) analysis of OM proteins from members of the *A. hydrophila* high-virulence serogroup stained with Coomassie blue. (A) Comparison of OM profiles of *A. hydrophila* TF7 prepared by sucrose density gradient centrifugation (lane 1) and sarcosyl digestion (lane 2). (B) Comparison of OM profiles of *A. hydrophila* strains prepared by sarcosyl digestion. Lanes: 1, TF7; 2, LL1; 3, A80-160; 4, A80-140; 5, P77-115; 6, A82-256. Molecular weight in thousands is given on the right.

peroxidase preformed complex (Amersham Corp., Arlington Heights, Ill.) was added at a 1:400 dilution in PBS-gelatin. The sheets were incubated for 1 h and then washed extensively with PBS. For color development we used 100 ml of PBS containing 50 mg of diaminobenzidine and 30 mg of $CoCl_2 \cdot 6H_2O$. The color appeared almost instantaneously upon addition of 1.0 ml of 30% H_2O_2, and the reaction was stopped by washing with a large volume of water.

Radioiodination of surface proteins. Cells were radioiodinated by using the Enzymobead System (Bio-Rad). Cells were suspended in 0.2 M phosphate buffer (pH 7.2) to an A_{550} of 10. A 1.0-ml sample of the undiluted suspension was used for radiolabeling as described in the Enzymobead procedure.

Radioiodination of glycine-extracted protein. Glycine-extracted protein was iodinated with ^{125}I by a modification of the procedure of Hunter and Greenwood (18). A 50-μg portion of protein in 100 μl of 0.5 M phosphate buffer (pH 7.0) was reacted with 1 mCi of ^{125}I and 100 μl of chloramine-T (2 mg/ml; Sigma Chemical Co., St. Louis, Mo.) in phosphate buffer for 3 min at room temperature. The reaction was stopped by the addition of 100 μl of sodium metabisulfite (4 mg/ml), 10 μl potassium iodide (10 mg/ml), and 1.0 ml 0.5% gelatin, all made up in phosphate buffer. Unbound ^{125}I was removed by dialysis.

Peptidoglycan-associated protein. Isolated OM (10 mg) was suspended in 1.0 ml of extraction buffer containing 2% (wt/vol) SDS, 10 mM Tris (pH 7.4), and 10% (vol/vol) glycerol and incubated at 60°C for 30 min by the method of Rosenbusch (33). Protein not solubilized by this treatment was peptidoglycan associated and was isolated by centrifugation at 45,000 × g for 1 h. The resulting pellet was washed and suspended in distilled water.

Assays. Protein was determined by the method of Markwell et al. (26) with bovine serum albumin as the standard. Total carbohydrate was measured by the phenol-sulfuric acid procedure of Dubois et al. (12) with glucose as the standard.

RESULTS

OM protein composition of high-virulence serogroup. Electron-microscopic examination of negatively stained preparations of *A. hydrophila* strains belonging to the high-virulence

serogroup described by Mittal et al. (28) showed that they all produced tetragonal S layers (R. G. E. Murray, J. S. G. Dooley, P. W. Whippey, and T. J. Trust, submitted for publication). As a first step toward identification of the S-layer subunit protein, we examined the surface protein composition of a selection of these strains by SDS-PAGE analysis of isolated OM fractions. To help ensure against artifacts or possible loss of components during processing, we first compared two methods of OM isolation from a representative of the high-virulence serogroup, *A. hydrophila* TF7. Very similar protein profiles were observed by both the sarcosyl digestion and sucrose density gradient methods (Fig. 1A). The sucrose density gradient-derived OM fraction of *A. hydrophila* TF7 had a density of 1.24 g/ml; however, this fraction also contained some extra, minor bands which appeared to result from slight contamination by inner membrane material as determined by an assay of NADH oxidase activity (results not shown). Assay of the sarcosyl-derived OM revealed almost no detectable NADH oxidase activity. The sarcosyl digestion method was therefore used for the remainder of the study, since this procedure was fast and convenient and appeared to produce an OM fraction with a minimum of inner membrane contamination.

OM material from members of the high-virulence group produced very similar electrophoretic profiles upon SDS-PAGE (Fig. 1B). In the *A. hydrophila* strains examined, major proteins of molecular weight 30,000 (30K proteins) and proteins in the molecular weight range of 45,000 to 55,000 were observed. This latter group of proteins was very poorly resolved in 12.5% acrylamide gels, however, appearing as a large smear. This effect was possibly due to the presence of LPS, which has been shown to migrate to the same area of the gel as the smeared proteins (9). In addition, UV-induced mutant *A. hydrophila* TF7/U14, which was deficient in the O-polysaccharide portion of its LPS, displayed no smearing of its major proteins (Fig. 2A and B, lanes 2). Significantly, despite the O-polysaccharide defect, this strain still produced an intact S layer which could be observed by electron microscopy. This was not the case with the deep rough LPS mutant, *A. hydrophila* TF7/B (Fig. 2A, lane 3). Electron microscopy showed that this mutant was unable to maintain an S layer on the cell surface, and SDS-PAGE analysis showed that during growth in liquid medium a 52K protein was excreted into the culture medium (Fig. 2C, lane 3).
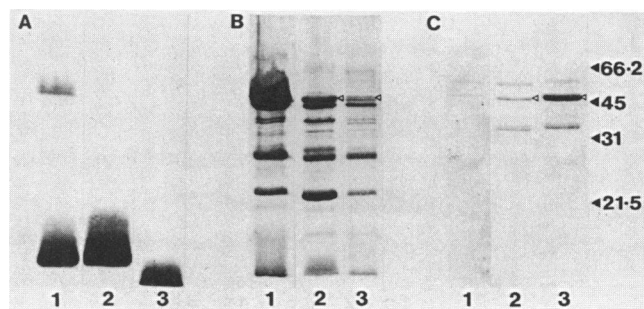

FIG. 2. SDS-PAGE (12.5% acrylamide) analysis of *A. hydrophila* TF7 (lanes 1) and the LPS mutants TF7/U14 (lanes 2) and TF7/B (lanes 3). (A) Silver stain of LPS from whole-cell lysates. (B) Protein profile of the sarcosyl-digested OM fraction stained with Coomassie blue. (C) Supernatant fluid after growth in static culture in tryptic soy broth for 24 h at 37°C, stained with Coomassie blue. The position of the S-layer protein is indicated (◁). Molecular weight in thousands is given on the right.
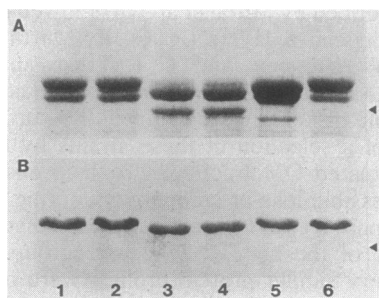
FIG. 3. (A) SDS-PAGE analysis of proteins in the OM fraction of members of the *A. hydrophila* high-virulence serogroup separated on 7.5% gels and stained with Coomassie blue. Lanes: 1, TF7; 2, LL1; 3, A80-160; 4, A80-140; 5, P77-115; 6, A82-256. (B) SDS-PAGE (12.5% acrylamide) of pH 3.0 glycine extracts of members of the *A. hydrophila* high-virulence serogroup stained with Coomassie blue. Lanes are the same as in panel A. A molecular weight marker is shown on the right at 45,000.

It was possible to obtain adequate resolution of the smeared region in wild-type strains by separating the proteins in the OM fraction on a 7.5% acrylamide gel (Fig. 3A). Under these separation conditions, strain TF7 was found to have three major proteins, the 48K, 50K, and 52K proteins. The 52K protein appeared to be the most abundant species. The other members of the high-virulence serogroup also possessed a similar group of three major proteins, but some differences in molecular weight were noted. For example, *A. hydrophila* A80.160 had a group made up of 44K, 47K, and 51K proteins, while strain P77.115 had a group made up of 47K, 50K, and 52K proteins.

Identification of the S-layer protein. Owing to the overall similarity in OM protein profiles of the members of the high-virulence group, strain TF7 was chosen for further analysis. After cells of TF7 were treated with glycine buffer at pH 3.0, the characteristic tetragonal S layer could not be demonstrated by electron microscopy. SDS-PAGE analysis of the glycine-extracted material showed that it was composed predominantly of a 52K protein (Fig. 4, lane 3). The extract also contained several minor proteins of lower molecular weight, and silver staining showed that the glycine extract also contained LPS (results not shown). Comparison of the OM from glycine-extracted cells with that from untreated cells further showed that a 52K protein was almost

completely removed from the cells by the glycine extraction procedure (Fig. 4, lane 2). It was noted that a residual amount of 52K protein was always left associated with the OM, even after repeated extractions.

Thus, the glycine extraction procedure released most of the 52K protein from the cell concomitant with the loss of the S layer. Analogous results were obtained when isolated OM was treated with the pH 3.0 glycine buffer (Fig. 4, lane 5). When the pH 3.0 glycine extraction procedure was applied to the other members of the high-virulence *A. hydrophila* serogroup, all the extracts contained primarily a protein of approximate molecular weight 52,000 (Fig. 3B). In every case this treatment also resulted in a loss of the ability to visualize the S layer by electron microscopy.

Extraction with glycine buffer at pH 4.0 caused the release of the S layer as sheets (Fig. 5A). The tetragonal patterning of this sheet material was indistinguishable from that of the native S layer on negatively stained whole cells. The sheet material was isolated by differential centrifugation, and SDS-PAGE analysis confirmed that the S-layer sheets were composed primarily of a protein of subunit molecular weight 52,000 (Fig. 5B). As with the lower-pH extract, the pH 4.0 extract also contained a small amount of LPS.

Surface topology. Further evidence that the 52K protein was the major S-layer subunit protein in TF7 was provided by immunochemical analysis. Immunoblot analysis with an antiserum that was raised to formalinized whole cells of TF7 and was absorbed with boiled cells of the same strain showed that this protein was clearly the major heat-labile antigen present on the cell, effectively masking other OM proteins (Fig. 6, lane 2). Additional confirmation of the masking of OM proteins by the S layer was provided by labeling surface-exposed lysine residues with Sulfo-NHS-Biotin. When the biotin-labeled proteins of TF7 were separated by SDS-PAGE, only a single major band of molecular weight 52,000 was seen on untreated cells (Fig. 6, lane 1).

The more penetrating lactoperoxidase surface-radiolabeling procedure was then applied to intact *A. hydrophila* TF7 cells before and after glycine extraction to identify OM proteins exposed on the outer surface of the OM under the S layer. When the lactoperoxidase technique was applied to untreated cells, a profile very different from that obtained by the surface biotinylation method was obtained. SDS-PAGE showed that five proteins (30K, 48K, 52K, 59K, and 80K
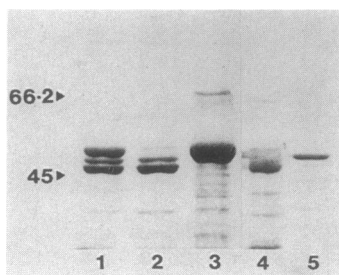

FIG. 4. SDS-PAGE (7.5% acrylamide) analysis of the effects of glycine extraction on whole cells and isolated OMs of *A. hydrophila* TF7, stained with Coomassie blue. Lanes: 1, OMs isolated from untreated cells; 2, OMs isolated from glycine-extracted cells; 3, glycine extract of whole cells; 4, OMs after extraction with glycine buffer; 5, glycine extract of isolated OMs. The samples in lanes 1 to 4 contained 20 μg of protein, and the sample in lane 5 contained 5 μg of protein. Molecular weight in thousands is given on the left.
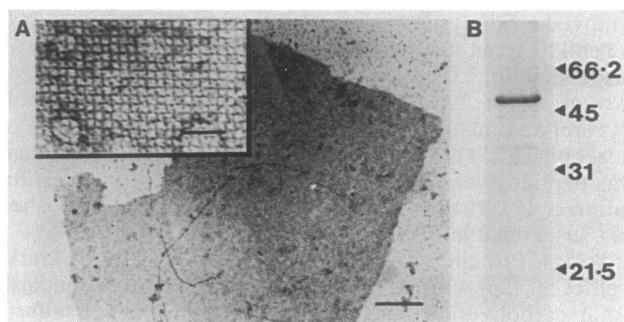

FIG. 5. Identification of the protein composition of the tetragonally arrayed sheet material released by treatment of *A. hydrophila* TF7 with glycine buffer (pH 4.0). (A) Sheet material negatively stained with 2% phosphotungstate (bar, 500 nm). The inset shows higher magnification of the tetragonal sheet material (bar, 50 nm). (B) SDS-PAGE gel (12.5% acrylamide) of isolated sheet protein stained with Coomassie blue. Molecular weight in thousands is given on the left.
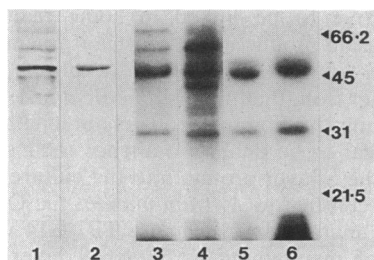
FIG. 6. Identification of surface-exposed proteins on *A. hydrophila* TF7 cells by using Sulfo-NHS-Biotin (lane 1), antiserum to whole cells (lane 2), and ^{125}I (lanes 3 to 6). Lane 1 shows a biotinylated 52K protein identified by horseradish peroxidase staining after SDS-PAGE (12.5% acrylamide) and transfer to nitrocellulose paper. Lane 2 shows an autoradiogram of an immunoblot of the OM of *A. hydrophila* TF7 reacted with antiserum (dilution, 1:500) prepared to whole cells of the same strain. Lanes 3 to 6 show autoradiograms of dried SDS-PAGE gels (12.5% acrylamide) of ^{125}I-labeled whole cells (lane 3) ^{125}I-labeled glycine-extracted whole cells (lane 4), OM fraction from ^{125}I-labeled whole cells (lane 5), and OM fraction from ^{125}I-labeled glycine-extracted cells (lane 6). Molecular weight in thousands is given on the right.

proteins) were labeled (Fig. 6, lane 3). The 52K protein was only weakly labeled. Cells were also radiolabeled after they had been extracted with 0.2 M glycine buffer (pH 3.0), which resulted in the labeling of a 43K protein that was not labeled in untreated cells (Fig. 6, lane 4). In addition, an increase in the amount of incorporation of the label into the 48K band was apparent. For non-glycine-extracted cells, the 30K, 48K, and 52K proteins copurified with the OM fraction (Fig. 6, lane 5). The 48K and 30K proteins were also present in the OM from the radiolabeled glycine-extracted cells (Fig. 6, lane 6). Interestingly, although the 52K S-layer protein was only weakly radiolabeled by the lactoperoxidase procedure in its native conformation on the cell surface, it could be labeled to a high specific activity by the chloramine-T procedure after it was washed off the surface with glycine buffer (data not shown).

Peptidoglycan association and heat modification of OM proteins. The 48K and 50K proteins present in the OM preparation remained associated with the peptidoglycan after extraction with SDS at 60°C (Fig. 7). At temperatures above 60°C these proteins were solubilized along with the other OM components. None of the proteins (52K, 30K, or any of the other minor proteins) displayed peptidoglycan association. Similar results were observed with the other members of the high-virulence group, although strains A80-160 and A80-140 differed slightly, having peptidoglycan-associated 47K and 44K proteins (results not shown).

The electrophoretic mobility of certain OM proteins, including porins, is markedly influenced by the temperature of solubilization in SDS (16, 33). The 50K and 48K proteins of *A. hydrophila* TF7 required a temperature of 60°C for solubilization (Fig. 7B). The mobility of these proteins changed dramatically at different solubilization temperatures; below 40°C they migrated as a high-molecular-weight aggregate at greater than 66,000. Between 40 and 60°C, although not seen in the area of the gel shown in Fig. 7A and B, this protein migrated with an apparent molecular weight of 35,000 and above 60°C the migration was indicative of a molecular weight of 48,000. The heat-modifiable mobility of the 48K and 50K proteins was the same irrrespective of whether they were removed from the OM (Fig. 7A and B). The SDS-PAGE mobility of the other major protein in the

OM fraction, the 30K protein, was not heat modifiable (data not shown). The mobility of these OM proteins was not affected by the presence or absence of β-mercaptoethanol, irrespective of the temperature of solubilization (data not shown).

The 52K S-layer protein associated with the OM appeared to enter the gel at temperatures above 40°C (Fig. 7A). However, when the *A. hydrophila* 52K protein was removed from the OM by extraction with 0.2 M glycine buffer (pH 3.0), its solubility was no longer temperature dependent (Fig. 7C). This behavior was different from that observed for the S-layer protein of *A. salmonicida*, which did not exhibit temperature-dependent solubility when associated with OM or after its release from the membrane following sodium deoxycholate solubilization (Fig. 7D and E).

DISCUSSION

A number of separate lines of evidence indicated that a 52K protein was the S-layer protein in *A. hydrophila* TF7. Removal of the 52K molecule from the cell surface by pH 3.0 glycine extraction resulted in the loss of a recognizable S layer as determined by electron microscopy. Indeed, tetragonal S-layer material in the form of intact sheets was readily seen during a less-denaturing pH 4.0 extraction of whole
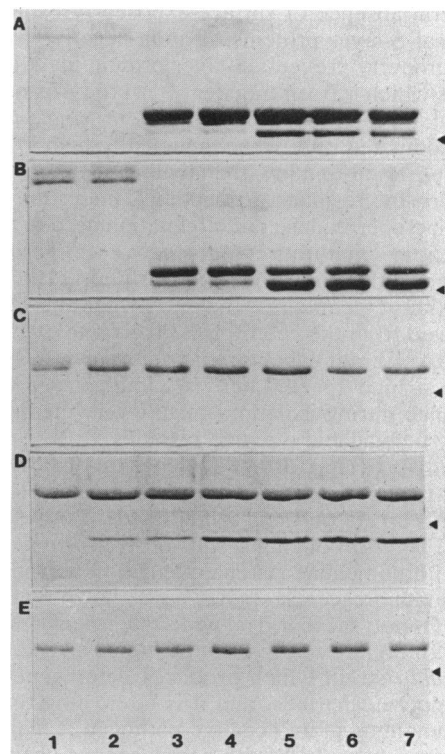

FIG. 7. Temperature-dependent SDS solubilization behavior of OM and S-layer proteins from *A. hydrophila* TF7 and *A. salmonicida* 449. Temperatures of solubilization: 30°C (lane 1), 40°C (lane 2), 50°C (lane 3), 60°C (lane 4), 70°C (lane 5), 80°C (lane 6), and 100°C (lane 7). (A) *A. hydrophila* TF7 OM fraction. (B) Heat-modifiable solubilization of 48K and 50K major OM proteins in peptidoglycan-associated protein fraction. (C) Solubilization of glycine-extracted 52K S-layer protein. (D) *A. salmonicida* 449 OM fraction. (E) Purified 49K S-layer protein of *A. salmonicida* 449. SDS-PAGE (7.5 acrylamide) stained with Coomassie blue. Molecular weight marker on right is at 45,000.

cells. These S-layer sheets were easily isolated by centrifugation, and SDS-PAGE analysis showed that the predominant protein in the S-layer sheet material was the 52K protein. This protein was also the major surface protein antigen on intact A. hydrophila TF7 cells. When assembled as an S layer, the 52K protein masked other major surface-exposed OM proteins, restricting access of a Sulfo-NHS-Biotin chemical label and shielding these OM proteins from immune recognition. Other members of the high-virulence A. hydrophila serogroup also produce a tetragonally arrayed S layer, and in each case SDS-PAGE analysis of glycine extracts indicated the presence of protein of molecular weight approximately 52,000. This molecular weight is consistent with the molecular weights reported for the tetragonal S-layer-forming proteins of other gram-negative bacteria, including the 60K S-layer protein of Azotobacter vinelandii (4), the 48K to 53K S-layer proteins of A. salmonicida (22), and the 65K putative S-layer protein of Pseudomonas sp. strain EU2 (14; unpublished data).

The Sulfo-NHS-Biotin impermeant chemical labeling procedure used to localize the S-layer subunit protein on the cell surface of A. hydrophila TF7 had previously been used to identify surface-exposed lysine residues in membrane proteins of bovine leukocytes and to confirm the surface location of the variable surface glycoprotein of certain African trypanosomes (15, 19). This sensitive method allowed us to circumvent the technical limitations imposed by the apparently general absence of surface-exposed tyrosine residues in tetragonal S-layer proteins of gram-negative bacteria (4, 22). This property prevents S-layer protein identification by techniques which rely on transfer of ^{125}I to tyrosine. Indeed, Bingle et al. (3) found that the Azotobacter vinelandii S layer was radioiodinated only when sufficiently high ^{125}I concentrations were used to allow the labeling of otherwise buried internal tyrosine residues. Predictably, under the mild conditions of lactoperoxidase radioiodination used in this study, there was little denaturation of the native A. hydrophila TF7 S layer, and so the S-layer protein was poorly labeled by the lactoperoxidase technique. However, when the 52K protein was removed from the cell by pH 3.0 glycine extraction and subjected to ^{125}I radiolabeling with chloramine-T, it could be labeled to a high specific activity, indicating a conformational change during isolation, resulting in the exposure of previously unavailable tyrosine residues.

This study provided further evidence that LPS structure is important to the ability of Aeromonas species to maintain tetragonal S layers on their cell surfaces. Both A. salmonicida and S-layer-producing strains of A. hydrophila produce an LPS with homogeneous-chain-length O polysaccharides, and immunofluorescence studies have shown that a number of these O polysaccharides penetrate their respective S layers (6, 9). So far, we have not identified an S layer on any A. hydrophila strain which produces heterogeneous-chain-length O polysaccharides, and it is interesting that another S-layer-containing gram-negative pathogen, Campylobacter fetus, also produces LPS with homogeneous-chain-length O polysaccharides (25), as do the tetragonal-S-layer-producing organisms Azotobacter vinelandii (11) and Pseudomonas sp. strain EU2 (14; unpublished data). Transposon mutagenesis in A. salmonicida has also provided evidence that the synthesis and export of S-layer protein are closely associated with the ability of the cell to produce homogeneous-chain-length O-polysaccharide-containing LPS (2). It would therefore seem that the association of this LPS morphotype with the presence of certain S layers is not merely fortuitous, and the synthesis, export, and maintenance of such S layers

may well prove to be linked in some manner to LPS O-polysaccharide production, export, or assembly.

The LPS–S-layer interaction in A. hydrophila TF7 does, however, differ from that in A. salmonicida. A. salmonicida mutants lacking the homogeneous-chain-length O polysaccharides appear to be unable to anchor their surface array and excrete the S-layer protein into the culture medium (2). However, in contrast to A. salmonicida, the O-polysaccharide-deficient mutant A. hydrophila TF7/U14 was found to be capable of maintaining an intact S layer on the cell surface. Immunofluorescence studies with a monoclonal antibody specific for the core oligosaccharide-polysaccharide linkage of the LPS of both species have shown that this linkage is exposed in A. salmonicida, but is masked by the S layer in A. hydrophila TF7 (9). The core oligosaccharide region of A. hydrophila TF7 LPS is also 2 residues longer than the core of A. salmonicida, so that proximity to the underlying OM would not appear to account for the ability of mutant TF7/U14 to maintain an S layer. However, there does appear to be a certain minimum LPS oligosaccharide size that is necessary to support an S layer, since A. hydrophila TF7/B, with its deep rough mutation in the core oligosaccharide, was unable to anchor its S layer and instead released the protein array into the growth medium.

When associated with the OM, the 52K A. hydrophila S-layer protein was released only when solubilized at >40°C. This contrasted with the SDS solubilization behavior of the A. salmonicida S-layer protein, which was readily solubilized without resort to heat, even when associated with the OM. This indicates that the A. hydrophila S layer has stronger interactions with OM components than the A. salmonicida S layer, although both Aeromonas layers appear to be more firmly stabilized than the morphologically similar S layer of Azotobacter vinelandii, which can be completely extracted by washing with distilled water (3). Indeed, repeated glycine extractions of A. hydrophila TF7 failed to completely remove all of the 52K protein from the OM fraction, indicating that a small number of S-protein molecules were very firmly associated with the OM.

Although the S layer restricted access of the Sulfo-NHS-Biotin label to underlying integral OM proteins, ^{125}I was able to penetrate through the S layer when the lactoperoxidase technique was used, and it could be used to radiolabel underlying OM proteins. This has also been shown to be the case with Azotobacter vinelandii (3) and A. salmonicida (22) and clearly reflects the greater accessibility of tyrosine residues in certain OM proteins. A 48K major OM protein was labeled by this procedure and exhibited characteristic SDS heat solubilization characteristics; it spanned the OM to be associated with peptidoglycanlike bacterial porin proteins (7, 16). A 50K major OM protein also exhibited these characteristics and may also represent a porin protein which lacks surface-exposed tyrosine residues. A 30K major protein was also present in all high-virulence-serogroup strains. In A. hydrophila TF7 this protein was exposed on the surface of the OM, but did not appear to be a transmembrane protein and was not peptidoglycan associated.

Interestingly, the SDS-PAGE protein profile of the OM fraction of all members of the high-virulence serogroup was very similar. This homogeneity of OM composition is in sharp contrast to the heterogeneity exhibited by the A. hydrophila strains described by Aoki and Holland (1). However, because these investigators did not examine a single serogroup and because of the recognized variation in habitat, host range, and virulence of A. hydrophila strains (8), such variation in OM protein composition is not unexpected. It is

tempting to speculate that this conservation of OM protein composition throughout the S-layer-containing group, together with the conservation of LPS structure which we previously reported (9), reflects special structural requirements for S-layer export, assembly, and maintenance. Certainly, S-layer-producing strains of *A. salmonicida* also display extensive homogeneity of OM protein composition, together with a structurally conserved LPS (9, 25).

This is the second identification of an S layer on a pathogen of salmonid fish. An S layer is ideally situated to afford a systemic pathogen, such as *A. hydrophila*, protection from host defense mechanisms. The S layer in *A. salmonicida* contributes to the ability of the cell to resist the serum killing activity of the host (29), and this also appears true for the *A. hydrophila* S layer (T. Sakata, J. S. G. Dooley, and T. J. Trust, unpublished). However, the types of disease produced by *A. hydrophila* and *A. salmonicida* are very different, suggesting that each organism may use its S layer in a different manner. Recent evidence indicates that S layers may be multifunctional proteins rather than simply inert barriers. For example, the S layer from *A. salmonicida* specifically binds porphyrins (23) and can also bind immunoglobulin (B. M. Phipps and W. W. Kay, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, K225, p. 231), properties which could be useful in helping the organism survive in vivo. Although these particular properties do not seem to be associated with the *A. hydrophila* S layer, it is likely that the S layer plays an important role in the pathogenesis of the high-virulence *A. hydrophila* serogroup. Further insight into the structure and function of this *A. hydrophila* protein should come about from studies of the biochemistry of the individual subunits.

ACKNOWLEDGMENTS

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